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Engineering *Clostridium acetobutylicum* for production of kerosene and diesel blendstock precursors

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ABSTRACT

Processes for the biotechnological production of kerosene and diesel blendstocks are often economically unattractive due to low yields and product titers. Recently, *Clostridium acetobutylicum* fermentation products acetone, butanol, and ethanol (ABE) were shown to serve as precursors for catalytic upgrading to higher chain-length molecules that can be used as fuel substitutes. To produce suitable kerosene and diesel blendstocks, the butanol:acetone ratio of fermentation products needs to be increased to 2–2.5:1, while ethanol production is minimized. Here we show that the overexpression of selected proteins changes the ratio of ABE products relative to the wild type ATCC 824 strain. Overexpression of the native alcohol/aldehyde dehydrogenase (AAD) has been reported to primarily increase ethanol formation in *C. acetobutylicum*. We found that overexpression of the AAD^{D485G} variant increased ethanol titers by 294%. Catalytic upgrading of the 824(aad^{D485G}) ABE products resulted in a blend with nearly 50 wt% $\leq C_9$ products, which are unsuitable for diesel. To selectively increase butanol production, *C. beijerinckii* aldehyde dehydrogenase and *C. ljungdhalii* butanol dehydrogenase were co-expressed (strain designate 824(*Cb ald-Cl bdh*)), which increased butanol titers by 27% to 16.9 g L^{-1} while acetone and ethanol titers remained essentially unaffected. The solvent ratio from 824(*Cb ald-Cl bdh*) resulted in more than 80 wt% of catalysis products having a carbon chain length $\geq C_{11}$ which amounts to 9.8 g L^{-1} of products suitable as kerosene or diesel blendstock based on fermentation volume. To further increase solvent production, we investigated expression of both native and heterologous chaperones in *C. acetobutylicum*. Expression of a heat shock protein (HSP33) from *Bacillus psychrosaccharolyticus* increased the total solvent titer by 22%. Co-expression of HSP33 and aldehyde/butanol dehydrogenases further increased ABE formation as well as acetone and butanol yields. HSP33 was identified as the first heterologous chaperone that significantly increases solvent titers above wild type *C. acetobutylicum* levels, which can be combined with metabolic engineering to further increase solvent production.

1. Introduction

Development of biotechnological processes for the production of fuel blendstocks has become a major research interest during the last decade. While processes for the production of short chain alcohols that can be blended with gasoline have been established, economically feasible routes to produce kerosene and diesel substitutes at sufficient titers via fermentation are still lacking (Choi and Lee, 2013; Howard et al., 2013; Steen et al., 2010). Recently, acetone and butanol or ethanol were shown to serve as raw materials for an alkylation reaction to form higher molecular weight gasoline, kerosene, and diesel blendstocks (Anbarasan et al., 2012). *C. acetobutylicum* is a well-suited organism for generating these reactants as it natively produces all three of them (Jones and Woods, 1986). A molar butanol:acetone ratio of 2–2.5:1 is required to fully convert the main *C. acetobutylicum* fermentation products to kerosene and diesel blendstocks (Sreekumar et al., 2014). Because the butanol:acetone ratio of wild type *C. acetobutylicum* ATCC 824 is only 1.6:1, increasing butanol production is an important goal. Ethanol production is undesirable as the short chain condensation products of acetone and ethanol are not suitable as diesel blendstocks. Furthermore, high

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acetone and butanol titers are necessary in order to reduce energy costs for recovery of the solvents and increase the economic viability of this process (Green, 2011).

Two different strategies to increase solvent titers in *C. acetobutylicum* have been previously explored: metabolic engineering to increase product titers and approaches to improve tolerance toward the solvents. Metabolic engineering efforts in *C. acetobutylicum* have mostly relied on increasing metabolite "push" towards butanol by deleting genes related to butyrate production (Harris et al., 2000), acetone production (Jiang et al., 2009), or a combination thereof (Jang et al., 2012). While this approach has resulted in the highest butanol titers and selectivities reported for *C. acetobutylicum* to-date, it is insufficient when acetone production is required, as is the case for catalytic upgrading of ABE products to kerosene and diesel blendstocks. Increasing metabolite "pull" towards butanol by overexpression of the native alcohol/aldehyde dehydrogenase (AAD) has been unsuccessful because it leaves butanol titers mostly unaffected and increases ethanol production (Tummala et al., 2003) except when both acetate and butyrate production are knocked out. In such acid-production defective strains with very high butanol fluxes, acetic and butyric acids are still produced and not completely re-assimilated, which results in loss of carbon to solvent products. To selectively increase butanol titers while preserving acetone production, more butanol-specific aldehyde and butanol dehydrogenases must be identified.

Increasing solvent tolerance by overexpression of the native chaperone complex GroE has also been shown to increase butanol production in *C. acetobutylicum*, as well as impart greater solvent tolerance (Tomas et al., 2003). Overexpression of GroE also resulted in a variety of regulatory or modulatory changes including a prolonged acidogenic phase due to delayed expression of solvent formation genes. Other attempts to increase solvent formation by chaperone expression have resulted in phenotypes with increased solvent tolerance but decreased solvent formation (Mann et al., 2012). Recently, heat shock protein 33 (HSP33) from solvent-tolerant *Bacillus psychrosaccharolyticus* was shown to increase solvent tolerance when expressed in *E. coli*. Since HSP33 is oxidatively activated, this chaperone could be useful to address oxidative stress caused by reactive oxygen species that form at high solvent titers (Zhu et al., 2011). Nonetheless, while both metabolic and tolerance engineering strategies have led to improved solvent producing clostridial strains, few studies have focused on simultaneously addressing the issues of solvent toxicity and metabolite flux bottlenecks.

In this report, we describe the identification of an aldehyde dehydrogenase and an alcohol dehydrogenase that selectively increase butanol production in *C. acetobutylicum*. We also show that expression of a heterologous heat shock protein improves solvent titers. The improved butanol:acetone ratio produced from our engineered strains allows the efficient downstream catalysis of ABE products to kerosene and diesel blendstocks with high yield. We also describe the first attempt to combine tolerance and metabolic engineering in *C. acetobutylicum* by combined expression of aldehyde/butanol dehydrogenases and a chaperone.

2. Material & methods

2.1. Chemicals, microorganisms, and plasmids

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzymes were purchased from New England Biolabs (Ipswich, MA) and used according to the manufacturer's instructions. *Clostridium acetobutylicum* ATCC 824 was purchased from American Type Culture Collection (Manassas, VA, USA). *Escherichia coli* TOP 10 was purchased from Invitrogen (Carlsbad, CA, USA). Plasmid pIMP1 and methylation plasmid pAN1 were kindly provided by Professor E. T. Papoutsakis (University of Delaware, DE, USA). Primers used and plasmids created in this work are described in the Supplementary material (S1, Suppl. Tables 1 and 2, respectively).

2.2. Strain maintenance

C. acetobutylicum cultures were inoculated using colonies from 2xYTG-agar plates (in $g\,L^{-1}$: tryptone 16, yeast extract 10, sodium chloride 4, glucose 5, agar 18, pH 5.8) and cultivated in clostridial growth medium (CGM, in $g\,L^{-1}$: glucose 80, yeast extract 5, potassium phosphate dibasic 0.75, potassium phosphate monobasic 0.75, ammonium acetate 2, magnesium sulfate heptahydrate 0.1, ferrous sulfate heptahydrate 0.01, manganese sulfate monohydrate 0.01, sodium chloride 1, L-cysteine hydrochloride monohydrate 0.5). Glycerol stocks were prepared with 35% glycerol and were stored at −80 °C. All precultures were inoculated from glycerol stocks (500 μL in 5 mL CGM supplied with 75 $\mu g\,mL^{-1}$ clarithromycin) and grown out overnight at 37 °C.

2.3. Assembly of expression plasmids

All plasmid assembly steps were carried out using isothermal DNA assembly as previously described (Gibson et al., 2009). The genes *C. beijerinckii ald* (uniprot ID: Q716S8), *C. acetobutylicum* ATCC 824 *groE, aad, bdhA, Lactobacillus brevis groE, Pseudomonas putida groE, Bacillus psychrosaccharolyticus hsp33*, *C. acetobutylicum* thiolase promoter, and *C. acetobutylicum* acetoacetate decarboxylase terminator were amplified from genomic DNA of the respective organisms and subsequently cloned into the pIMP1 plasmid. Hexa-histidine tags were added by whole plasmid PCR with the respective primers. *C. ljungdahlii* butanol dehydrogenase, *bdh*, (uniprot ID: D8GL45) was codon optimized for expression in *C. acetobutylicum* (Supplementary material, S1) and cloned into pIMP1. Plasmid assembly is further described in the supplementary material. All PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) prior to the isothermal DNA assembly reaction.

2.4. DNA isolation and manipulation

pIMP1 was propagated in *E. coli* TOP10 (Invitrogen). All plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen). *E. coli* TOP10 was transformed using the standard heat shock protocol. Colonies were grown on LB-agar plates containing 100 $\mu g\,mL^{-1}$ carbenicillin. Plasmids were methylated by co-transformation with pAN1 in *E. coli* TOP10 and grown on LB-agar plates supplied with 100 $\mu g\,mL^{-1}$ carbenicillin and 50 $\mu g\,mL^{-1}$ chloramphenicol. Transformation of *C. acetobutylicum* was carried out as described (Mermelstein and Papoutsakis, 1993).

2.5. Fermentation

Fermentations were carried out at 37 °C in 3-L bioreactors (Bioengineering AG, Switzerland) with a working volume of 2 L. Seed-cultures (100 mL CGM, 75 $\mu g\,mL^{-1}$ clarithromycin, in sealed 150 mL anaerobic flasks) were inoculated with 100 μL preculture and grown to an OD_{600} of 2.0. Fermentation cultures were inoculated with 60 mL seed culture. Reactor medium CGM was supplied with 75 $\mu g\,mL^{-1}$ clarithromycin. The pH was controlled ≥ 4.9 with 5 M KOH. Glucose was added with a concentrated solution (in $g\,L^{-1}$: glucose 600, yeast extract 60) when the concentration of glucose dropped below 20 $g\,L^{-1}$. Plasmid presence was confirmed by PCR for seed cultures and after 30 h of the fermentation.

2.6. Butanol growth tolerance

Hungate anaerobic tubes containing 5 mL CGM supplemented with butanol at 0, 4, 10, 14, or 16 g L^{-1} were inoculated to an OD_{600} of 0.02 from precultures in mid-exponential growth phase (OD_{600} 0.4 − 0.8). Anaerobically sealed tubes were incubated at 37 °C and OD_{600} was recorded every 45 min using a Spectronic 200+ tube photometer (Thermo Scientific, Waltham, MA, USA) until the fastest growing cultures reached an OD_{600} of 1.0. Experiments were carried out in triplicate, and growth rates were calculated for individual experiments and subsequently averaged.

2.7. Alkylation of acetone

In a 12 mL Q-tube containing a stir bar and 2 wt% Pd-HT, acetone, ethanol, butanol, and dodecane (internal standard) were sequentially added. The Q-tube was sealed and the reaction mixture was stirred for 5 h at 250 °C in a preheated metal block after which the reaction mixture was cooled to room temperature. The reaction mixture was diluted with tetrahydrofuran and the GC analysis of the crude product mixture was carried out. Butanol, acetone, ethanol, and Pd-HT masses were chosen based on the ABE ratio from the respective fermentations (in mg): 148.0, 55.7, 130.0, 150.0 for 824(*aad*^{D485G}); 168.7, 53.9, 35.9, 145.0 for 824(*Cb ald-Cl bdh*); and 133.2, 52.2, 33.1, 141.0 for 824(*pIMP*) control.

2.8. Analytical methods

The expression of recombinant proteins was assessed using SDS-PAGE and Western blot analysis per the manufacturer's instructions for each technique. Metabolite concentrations for glucose, acetate, butyrate, ethanol, acetone, and butanol were determined using a Shimadzu Prominence HPLC System (Shimadzu, Kyoto, Japan) with UV/vis and refractive index detectors and equipped with a Bio-Rad (Hercules, CA, USA) Aminex HPX-87H ion exchange column with a Cation H guard column. Analysis was carried out using 5 mM sulfuric acid as the mobile phase with a flow rate of 0.7 mL min^{-1} at 35 °C. Metabolite concentrations were calculated using a 6-point external standard calibration. GC analysis was performed on a Varian CP-3800 instrument with an FID detector and VF-5 MS column (5% phenyl and 95% methylpolysiloxane) using helium as the carrier gas.

3. Results

3.1. Selective butanol production

To efficiently produce kerosene and diesel blendstocks from downstream chemical catalysis, the butanol:acetone ratio needs to be increased above wild type levels without the formation of additional ethanol. Overexpression of a mutant aldehyde/alcohol dehydrogenase AAD^{D485G} has been shown to improve butanol production in *C. acetobutylicum* when acid production genes are knocked out (Jang et al., 2012). However, knocking out acid production genes prevents formation of acetone, which is an essential reactant for the downstream alkylation reactions. Therefore, we investigated if similar effects could be observed in the context of an unaltered metabolism. Strain 824(*aad*^{D485G}) produced 14.8 g L^{-1} butanol, 5.54 g L^{-1} acetone, and 13 g L^{-1} ethanol (Fig. 1a). By comparison, a control strain with a non-expressing pIMP1 plasmid (strain 824(*pIMP*)) produced 13.3 g L^{-1} butanol, 5.1 g L^{-1} acetone, and 3.3 g L^{-1} ethanol (Supplementary material). The increase of 11% in butanol and 294% in ethanol titers clearly demonstrates that butanol titers cannot be selectively increased with the AAD^{D485G} variant when butyric and acidic acid production pathways are active. More butanol-specific aldehyde and alcohol dehydrogenases are likely required to selectively increase butanol titers while maintaining acetone production.

An amino acid similarity search of the aldehyde dehydrogenase portion of AAD identified the 25% similar aldehyde dehydrogenase from *C. beijerinckii* (ALD), which is reported to be more active towards butyryl-CoA than acetyl-CoA (Toth et al., 1999). To identify a potential butanol dehydrogenase, butanol dehydrogenase sequences I and II from *C. acetobutylicum* were queried against the NCBI Nucleotide collection. A predicted NAD-dependent butanol dehydrogenase from *C. ljungdahlii* was identified (BDH). Both enzymes were expressed under control of the constitutive thiolase promoter in a polycistronic operon in strain 824(*Cb ald-Cl bdh*). SDS-PAGE analysis revealed that both enzymes were expressed, albeit the amount of BDH was significantly higher (supplementary material). Strain 824(*Cb ald-Cl bdh*) produced 16.9 g L^{-1} butanol, 5.4 g L^{-1} acetone, and 3.6 g L^{-1} ethanol after 50 h (Fig. 1b). In contrast, expression of *C. acetobutylicum* butanol dehydrogenase A in combination with *Cb ald* (strain 824(*Cb ald-Ca bdh*) did not increase butanol titers above wild-type level (Supplementary material). Compared to the control strain 824(*pIMP*), butanol titers of strain 824(*Cb ald-Cl bdh*) were 27% higher, while acetone and ethanol titers were unaffected. Therefore, in contrast to AAD overexpression, heterologous aldehyde and butanol dehydrogenases led to a selective increase in butanol titers without increased ethanol formation.

3.2. Catalytic upgrading of ABE fermentation products to biofuel blendstocks

The solvent ratio of *C. acetobutylicum* fermentation products largely influences the product distribution in downstream catalysis. To demonstrate the requirement of a high butanol:acetone ratio for the production of diesel and kerosene blendstocks, we used solvent mixtures with ratios according to fermentations with the control strain, 824(*aad*^{D485G}), and 824(*Cb ald-Cl bdh*) for the alkylation of acetone (Fig. 2). The chain length distribution obtained with the solvent ratio of 824(*aad*^{D485G}) was centered around C_9 and C_{11} products while significant amounts of $\leq C_7$ products were present. The low ethanol concentration and high butanol:acetone ratio produced by 824(*Cb ald-Cl bdh*) resulted in a shift to longer chain-length catalysis products. The C_{11} products showed the highest abundance while the amount of $\leq C_7$ products that are unsuitable as diesel blendstock could be significantly reduced. The distribution of products from 824(*Cb ald-Cl bdh*) and control-strain ABE ratios were similar, but $\geq C_{11}$ alkylation product concentration (9.8 g L^{-1} based on fermentation volume) from the 824(*Cb ald-Cl bdh*) solvent products was 24% higher (Supplementary material). These results show that diesel blendstocks can be effectively produced by chemical catalysis using *C. acetobutylicum* fermentation products with a high butanol:acetone ratio and low ethanol concentration. The selective increase of butanol production could not be achieved by overexpressing the native alcohol/aldehyde dehydrogenase but was possible by expressing heterologous aldehyde and butanol dehydrogenases.

3.3. Combined expression of GroE and aldehyde/butanol dehydrogenases

Downstream processing represents a major cost associated with the ABE fermentation. Increasing product titers can significantly reduce these costs. Overexpression of the chaperonin complex GroE in *C. acetobutylicum* has been shown to increase solvent tolerance and result in increased solvent titers (Tomas et al., 2003). In order to further increase the solvent titers of strain 824(*Cb ald-Cl bdh*), butanol-specific dehydrogenases were

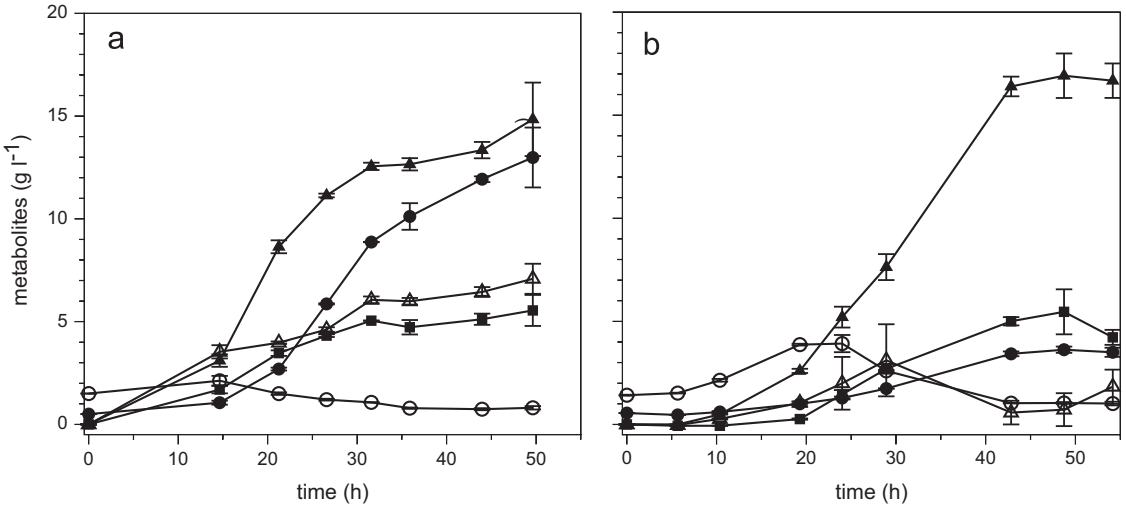

Fig. 1. Product concentrations of 824(*aad*^{D485G}) (a) and 824(*Cb ald-Cl bdh*) (b) during fermentation. Metabolites: acetic acid ○, butyric acid △, ethanol ●, butanol ▲, acetone ■. Three independent samples were taken and analyzed for every time point; error bars show standard deviations of the three measurements. For biological replicates see Supplementary material.

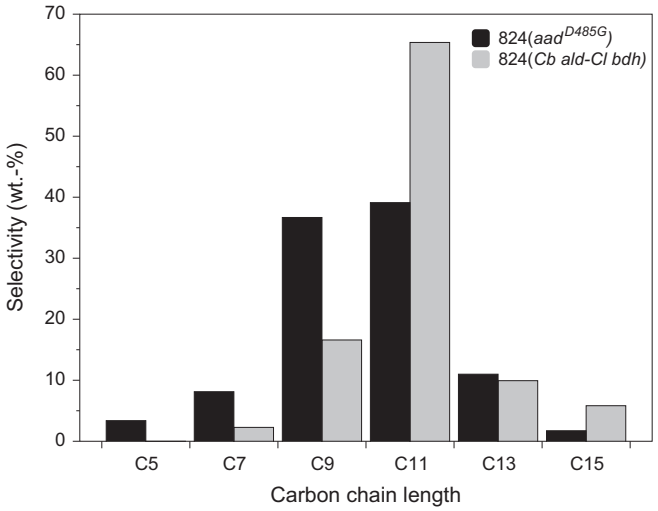

Fig. 2. Chain length distribution of alkylation reaction products obtained with solvent ratios of strains 824(*aad*^{D485G})) and 824(*Cb ald-Cl bdh*). Absolute mass data are provided in the Supplementary material.

expressed in combination with the native GroE. Strain 824(*Ca groE*), overexpressing only homologous GroE, was also investigated for comparison, and produced solvent titers of 15.2 g L^{-1} butanol, 5.1 g L^{-1} acetone, and 5.6 g L^{-1} ethanol in 43 h (Fig. 3a). By comparison, 824(*Cb ald-Cl bdh-Ca groE*) produced 14.3 g L^{-1} butanol, 5.1 g L^{-1} acetone, and 4.5 g L^{-1} ethanol (Fig. 3b). Compared to either 824(*Ca groE*) or 824(*Cb ald-Cl bdh*) these titers represent a reduction in solvent production. Since overexpression of GroE has been shown to have substantial regulatory implications such as a delayed solventogenic switch (Tomas et al., 2003), we hypothesized that simultaneous expression of GroE and aldehyde/butanol dehydrogenases might have counteractive effects. To circumvent these limitations, we sought to identify heterologous chaperones that are not detrimental to solvent production when expressed in combination with butanol and aldehyde dehydrogenases.

In addition to the native GroE, we also investigated the GroE homologs from the solvent-tolerant organisms *Lactobacillus brevis* and *Pseudomonas putida*. SDS-PAGE analysis of 824(*Pp groE*) and 824(*Lb groE*) did not reveal expression of the respective chaperones (Supplementary material). Moreover, protein expression of his-tagged *Lb* GroE and *Pp* GroE could not be detected with a Western blot analysis. Strains 824(*Pp groE*) and 824(*Lb groE*) grew poorly and did not switch from acid production to solvent production (data not shown). Therefore, expression of heterologous GroE could not be verified and the respective strains produced little solvent.

3.4. Expression of heterologous heat shock protein 33

Because of the poor performance of our engineered strains overexpressing heterologous GroE complexes, we investigated a different class of chaperones. HSP33 from *Bacillus psychrosaccharolyticus* has recently been shown to confer solvent tolerance when expressed in *E. coli* (Kang et al., 2007). Therefore, we investigated whether this protein could enhance solvent tolerance and formation when constitutively expressed in *C. acetobutylicum*. While SDS-PAGE followed by Coomassie staining did not reveal a band for HSP33, expression of the N-terminally hexa-histidine-tagged variant was verified by Western blot analysis (Supplementary material). Further experiments were carried out with the his-tagged protein. Strain 824(*Bp hsp33*) produced 15.7 g L^{-1} butanol, 6.1 g L^{-1} acetone, and 4.7 g L^{-1} ethanol after 49 h (Fig. 4a) – an increase of 18%, 19%, and 42% in butanol, acetone, and ethanol titers, respectively, compared to the plasmid control strain.

Expression of his-tagged HSP33 increased solvent titers to concentrations above those that we observed from strain 824(*Ca groE*). Native GroE overexpression has been shown to increase growth rates at elevated butanol concentrations; thus we examined whether strain 824(*Bp hsp33*) showed similar characteristics. Plasmid control strain 824(*pIMP*) and 824(*Bp hsp33*) were grown at butanol concentrations between 0 and 16 g L^{-1}. Growth inhibition for both strains was virtually identical (Supplementary material). In the absence of butanol, both strains grew with a growth rate of 0.54 h^{-1}. Unlike overexpression of native GroE, expression of HSP33 did not confer increased solvent tolerance during growth.

3.5. Combined expression of heat shock protein HSP33 and aldehyde/butanol dehydrogenases

Because co-expression of GroE and aldehyde/butanol dehydrogenases showed detrimental effects on solvent production, we examined the co-expression of heterologous HSP33 and aldehyde/butanol dehydrogenases. Strain 824(*Cb ald-Cl bdh-Bp hsp33*) produced 16.3 g L^{-1} butanol, 6.4 g L^{-1} acetone, and 5.6 g L^{-1} ethanol

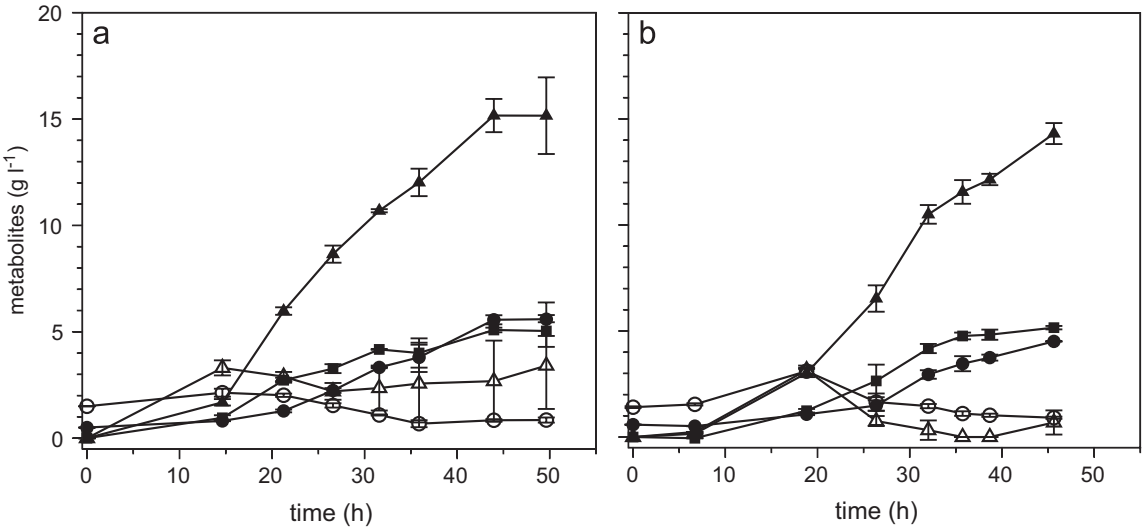

Fig. 3. Product concentrations of 824(*Ca groE*) (a) and 824(*Cb ald-Cl bdh-Ca groE*) (b) during fermentation. Metabolites: acetic acid ○, butyric acid △, ethanol ●, butanol ▲, acetone ■. Three independent samples were taken and analyzed for every time point; error bars show standard deviations of the three measurements. For biological replicates see Supplementary material.

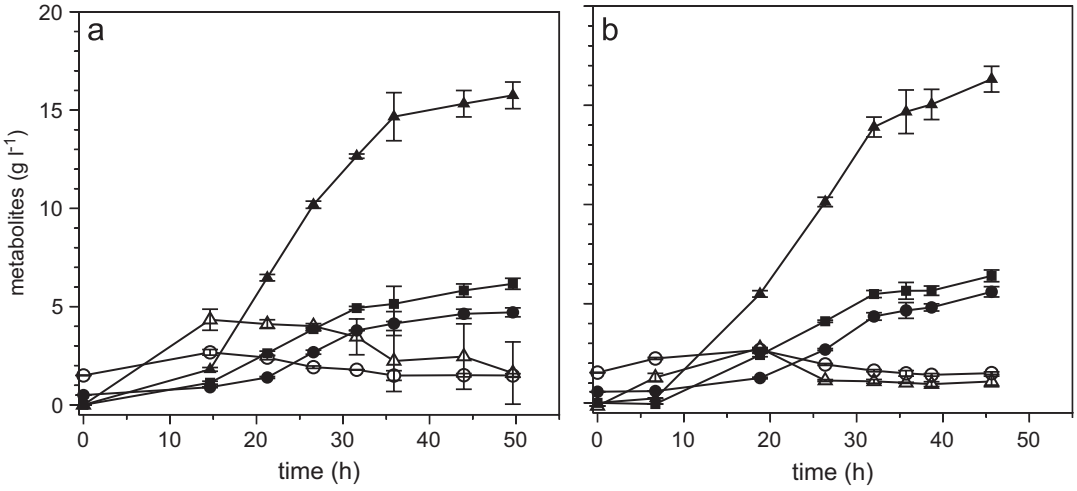

Fig. 4. Product concentrations of 824(*Bp hsp33*) (a) and 824(*Cb ald-Cl bdh –Bp hsp33*) (b) during fermentation. Metabolites: acetic acid ○, butyric acid △, ethanol ●, butanol ▲, acetone ■. Three independent samples were taken and analyzed for every time point; error bars show standard deviations of the three measurements. For biological replicates see Supplementary material.

after 45 h (Fig. 4b). In contrast to 824(*Cb ald-Cl bdh-Ca groE*), which produced less of each solvent than 824(*Ca groE*), the combined expression of aldehyde/butanol dehydrogenases and HSP33 increased total solvent formation compared to expression of only HSP33. Comparing the protein expression of 824(*Cb ald-Cl bdh-Ca groE*) and 824(*Cb ald-Cl bdh-Bp hsp33*) via SDS-PAGE indicates that these strains exhibited distinctly different protein expression patterns throughout the fermentation (Supplementary material). Specifically, 824(*Cb ald-Cl bdh-Bp hsp33*) showed similar expression levels of *C. ljungdahlii* butanol dehydrogenase and *C. beijerinckii* aldehyde dehydrogenase over the course of the fermentation. In contrast, 824 (*Cb ald-Cl bdh-Ca groE*) exhibited strong expression of both dehydrogenases and GroEL in the beginning while the relative levels of these proteins decreased over the course of the fermentation. Presence of the plasmid was verified by PCR to ensure that loss of plasmid was not responsible for the observed effect. Moreover, presence of antibiotic should have provided sufficient selection pressure as *C. acetobutylicum* cultures carrying pIMP or overexpressing *Ca groE* have been shown to retain plasmids even in the absence of antibiotic (Tomas et al., 2003).

3.6. Comparison of solvent ratios and yield

The primary aim of this study was to generate *C. acetobutylicum* strains that produced butanol and acetone at a molar ratio of 2–2.5:1 at increased titers with reduced ethanol production. Strain 824 (*aad*^{D485G}) showed the lowest butanol and acetone yield due to excessive ethanol production (Table 1). In contrast, butanol titers could be selectively increased by expression of heterologous aldehyde and butanol dehydrogenases while preserving acetone production. This resulted in the production of 16.9 g L^{-1} of butanol at a butanol: acetone ratio of 2.45:1, which is the ratio needed for downstream chemical catalysis to long-chain products that are suitable as diesel blendstocks. The increased butanol selectivity carried over to combined chaperone-dehydrogenase expressing strains even in the case of 824(*Cb ald-Cl bdh-Ca groE*), which did not produce solvent titers as high as 824(*Ca groE*) or 824(*Cb ald-Cl bdh*). Expression of HSP33 both alone and in combination with aldehyde and butanol dehydrogenase increased the overall solvent titers. Specifically, 824(*Cb ald-Cl bdh –Bp hsp33*) showed the highest combined butanol and acetone yield and produced the highest combined butanol + acetone titer (Table 1).

Table 1
Comparison of solvent production and butanol selectivity. Theoretical butanol and acetone yields are 1 mol (mol glucose)$^{-1}$. BuOH: butanol, AcO: acetone. Values include the standard deviation of three independent samples.

Strain 824(…)	Glucose consumed (g L^{-1})	Solvents (g L^{-1})	Acetone (g L^{-1})	Butanol (g L^{-1})	Ethanol (g L^{-1})	Yield BuOH+AcO (% theor.)	Molar ratio BuOH:AcO
pIMP	68	21.7	5.1 ± 0.1	13.3 ± 0.1	3.3 ± 0	71.8	2.04
Ca groE	79	25.9	5.6 ± 0.1	15.2 ± 0.8	5.6 ± 0.2	68.8	2.13
Bp hsp33	85	26.5	6.1 ± 0.3	15.7 ± 0.7	4.7 ± 0.2	67.2	2.02
aad^{D485G}	96	33.3	5.5 ± 0.8	14.8 ± 1.8	13.0 ± 1.5	55.2	2.11
Cb ald-Cl bdh	82	25.9	5.4 ± 1.1	16.9 ± 1.1	3.6 ± 0.2	70.5	2.45
Cb ald-Cl bdh-Ca groE	71	23.9	5.1 ± 0.1	14.3 ± 0.5	4.5 ± 0	71.2	2.20
Cb ald-Cl bdh-Bp hsp33	80	28.3	6.4 ± 0.3	16.3 ± 0.6	5.6 ± 0.3	74.3	2.00

4. Discussion

The production of kerosene and diesel blendstocks by alkylation of acetone using *C. acetobutylicum* fermentation products requires a high butanol:acetone ratio and little ethanol production. The expression of AAD^{D485G} increased the ethanol/butanol ratio and confirms the results of Harris et al. (2000), who showed that overexpression of native AAD results in only a modest increase in butanol but a much greater increase in ethanol titers. These results differ from those for an acid-production defective strain in which AAD^{D485G} overexpression mainly increased butanol production (Jang et al., 2012). Thus, overexpression of AAD^{D485G} in the context of an unaltered clostridial metabolism, as is the context of this work, increased ethanol production, indicating that this variant does not exhibit significantly greater selectivity for butanol than for ethanol compared to the native enzyme.

All strains expressing heterologous butanol and aldehyde dehydrogenase showed increased selectivity towards butanol as well as higher butanol titers. Ethanol titers were similar to plasmid control strain concentrations, which is probably due to the activity of native AAD. Knocking out the native AAD in addition to expressing the *C. beijerinckii* aldehyde dehydrogenase and *C. ljungdahlii* butanol dehydrogenase used in this work could substantially reduce ethanol production, which is crucial to maximize the yield of n-butanol and acetone. Acetone production was present in all strains studied, indicating that the increase in butanol titer was mainly a result of increased pull towards butanol due to butanol and aldehyde dehydrogenases. This finding is in contrast to studies involving the deletion of acid production pathways, which increased butanol titers mainly due to an increased push from acetoacetyl-CoA towards butanol (Harris et al., 2000; Jang et al., 2012). The introduction of these heterologous dehydrogenases thus presents a different rationale for metabolic engineering that can be further combined with knocking out acid production pathways to increase butanol titers. It also represents a viable strategy to increase butanol titers while maintaining acetone production, which is crucial for the downstream catalytic upgrading of these reactants to advanced biofuel molecules.

Co-expression of aldehyde and butanol dehydrogenase with the chaperone GroE was investigated as a way to further increase solvent formation by combining metabolic and solvent tolerance engineering. The resulting decrease in solvent formation is believed to stem from GroE regulatory effects. GroE overexpression delays the switch to solventogenesis, which is counteracted by constitutive expression of aldehyde/butanol dehydrogenases. As a major negative modulator of the CIRCE heat shock regulons GroE overexpression also reduces expression of other heat shock proteins, which could in turn exert a negative influence when combined with the early expression of butanol production genes (Tomas et al., 2003). As such, GroE overexpression may counteract metabolic engineering strategies that seek to increase solvent productivity by constitutive expression of solvent formation genes.

Expression of HSP33 resulted in increased solvent titers that were above those of a strain expressing GroE. Furthermore, the co-expression of aldehyde/butanol dehydrogenase and HSP33 increased solvent formation compared to expression of HSP33 alone and did not have detrimental effects on solvent production. While total solvent titers were higher than those of a strain expressing only aldehyde and butanol dehydrogenases, the butanol concentration was slightly lower. Lack of additional butanol production was presumably due to the low expression level of the chaperone, which could only be detected by Western blot and not by SDS-PAGE. As an increase in GroE expression level has been shown to increase butanol tolerance and solvent formation in *C. acetobutylicum*, optimizing the expression of HSP33 will likely lead to a further increase in solvent production as well (Tomas et al., 2003). HSP33 expression did not confer any observable growth-related butanol tolerance whereas Kang et al. (2007) clearly showed increased solvent tolerance of *E. coli* expressing this protein. This finding differs from results obtained for chaperones GroE, GrpE, and HtpG (Mann et al., 2012) in which these proteins increased solvent tolerance, yet their expression did not necessarily improve solvent production. Thus, screening for growth-associated tolerance will not necessarily identify organisms with enhanced solvent production capabilities.

Chaperone expression and metabolic engineering are both promising routes to increase solvent production in *C. acetobutylicum*. Expression of HSP33 increased solvent formation in *C. acetobutylicum* and can be used in a combined tolerance and metabolic engineering approach to enhance solvent production. The results presented here will be useful for both metabolic engineering and solvent tolerance engineering efforts in *C. acetobutylicum* and they clearly demonstrate that the use of heterologous proteins can help overcome limitations of native enzymes. The butanol and aldehyde dehydrogenases described in this work enable the selective increased production of butanol while acetone production is maintained. By altering the ratio of Clostridial fermentation products through metabolic engineering to more closely align with those required for optimal catalysis, kerosene and diesel blendstocks can be produced from biomass sugars at high selectivities and overall process yields.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2014.07.003.

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